GENOTOXIC EFFECTS OF OESTROGENS AND NANO-NSAIDS

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GENOTOXIC EFFECTS OF OESTROGENS AND NANO-NSAIDS:

Genotoxic effects of oestrogens *in vivo* and nano- and bulk forms of NSAIDs on blood samples from prostate cancer patients.

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ABSTRACT

The genotoxicological effects of five intra-peritoneal administered oestrogens (17β-oestradiol, daidzein, diethylstilboestrol, genistein, and equol), were examined. Male hooded-Lister rats were used to examine to what extent DNA damage occurred. The alkaline Comet assay was the chosen method used to assess double-strand DNA breakage by examining the Olive tail moment and %age tail DNA. Tissues from the testis, bone marrow, liver and blood were analysed after an 8-day duration of exposure. Statistically significant increases in DNA damage were observed in the testis with daidzein and in the blood with diethylstilboestrol.

In addition, a further study was carried out to examine the effects of bulk and nanotised forms of non-steroidal anti-inflammatory drugs (NSAIDs), aspirin and ibuprofen, in the Comet and micronucleus assays, on whole blood taken from prostate cancer patients or volunteers. These were used because it is known that the sensitivity of DNA to genotoxins can be heightened in patients with cancer. Patients' and volunteers' blood was cultured with either the bulk or nano-forms for 44 hours at 37°C, 5% CO_2. Data were obtained for the Comet assay as above and the number of binucleated cells scored for the micronucelus assay. The results show the nanotised forms of the NSAIDs decreased the levels of strand breakage and lowered the numbers of micronuclei generated compared with their bulk forms. There was no clear difference between the sensitivity of the healthy controls and the prostate cancer patients, with only one individual showing evidence of heightened sensitivity.

Keywords: **hooded-Lister rats**, **Oestrogens**, **Aspirin**, **Ibuprofen**, **Nano form**, **Blood**, **Comet assay**, **Micronucleus assay**, **Prostate cancer**.

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To my wonderful wife and daughter, there are no words I can use to express my gratitude and love for all of your continued support.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ASP B	Aspirin bulk form
ASP N	Aspirin nano-form
BER	Base excision repair
BiNC	Binucleated
CBMN	Cytokinesis block Micronucleus assay
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
DES	Diethylstilboestrol
EDTA	Ethylene diamine tetra acetic acid
GI	Gastrointestinal
GG-NER	Global genomic nucleotide excision repair
GPX	Glutathione peroxidase
hL	Hooded-Lister rats
IBU B	Ibuprofen bulk form
IBU N	Ibuprofen nano-form
IPCS	International Programme on Chemical Safety
i.p.	Intra-peritoneal
MNi	Micronuclei
MN	Micronucleus assay
MonoNC	Mononucleated
NAG-1	NSAID activating gene-1
NCI	National cancer institute
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
NDI	Nuclear division index
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffer solution
PHA	Phytohaemaglutinin
PTGS	Prostaglandin-endoperoxide synthase
5072PC	Prostate cancer patient
5169PC	Prostate cancer patient
5329PC	Prostate cancer patient
PSA	Prostate specific antigen
ROS	Reactive oxygen species
S.E.M	Standard error of the mean
SOD	Superoxide dismutase
5081SPC	Suspected prostate cancer patient
5082SPC	Suspected prostate cancer patient
TNF-α	Tumour necrosis factor alpha
WHO	World Health Organisation

Hospital Label

Influence on susceptibility in vitro and DNA damage.

FOR CLINICAL USE ONLY DATE OF SAMPLE.....

Hospital No..... CONSENT: YES/ NO

GENERAL INFORMATION

GENDER: MALE/ FEMALE

ETHNIC GROUP:.....

AGE:..... WEIGHT:.....

OCCUPATION.....

CURRENT SMOKER Y/N PAST SMOKER Y/N

HOW MANY PER DAY/WEEK?.....

SMOKING TYPE: CIGARETTES CIGARS PIPE

ALCOHOL YES/ NO

UNITS CONSUMED PER WEEK/DAY:.....

TYPE OF DIET: WESTERN ASIAN VEGTARIAN VEGAN

VITAMIN SUPPLEMENTS.....

PRESCRIBED MEDICATION.....

DIAGNOSIS.....

DATE.....

OTHER MEDICAL CONDITIONS.....

FAMILY HISTORY OF CANCER.....

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Paper submitted for publication:

Rathore. D.S., Cemeli, E., Neill, J., Grayson, B., Anderson, D. (2014). The effects of five oestrogenic compounds on DNA damage in the Comet assay in male hooded-Lister rats. *Mutational Research/ Genetic Toxicology and Environmental Mutagenesis.*

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Society demands that technologies evolve and become more efficient and financially viable. This has forced current manufacturing technologies, especially in cosmetics and packaging, to incorporate compounds with oestrogen-like properties into products as plasticisers. These have been implicated in increased levels of exposure and have been alleged to cause health associated problems (Chakraborty et al., 2012, Macon and Fenton, 2013, Molokhia and Perkins, 2008, Darbre, 2006, Rousseaux and Schachter, 2003). This advancement has now also encompassed nano-technology, which has preceded nano-toxicology, which thus lags behind. Therefore it is essential to examine the toxicological effects of these technologies. Examining engineered nano-particles using the Comet and micronucleus assays is an approach that should be used to assess the potential genotoxic effects of nano- forms of chemicals (Magdolenova et al., 2014).

1.1.1 Oestrogenic compounds

Oestrogenic compounds play a critical role in the pathophysiology of some diseases, particularly in the development of cancer. The types of oestrogen-dependent cancers vary depending on the tissue however breast cancers and endometrial cancers show high prevalence in women in the Western World. Emerging data seems to suggest a detrimental effect in men accounting for the oligozoospermia, which seems to be more evident in places such as Scandinavia (Tsourdi et al., 2009).

Oestrogenic compounds possess the ability to cause DNA lesions via their metabolism, which produces reactive oxygen species (ROS). ROS compounds induce oxidative stress and consequently cause DNA lesions and aberrations (Anderson et al., 2003). This can lead to modifications both directly to the DNA (8-oxo-guanine), and as a result of enzymatic repair mechanisms (base excision repair, global genome nucleotide excision repair and

transcriptional-coupled nucleotide excision repair mechanisms). The inability for accurate repair and the constant onslaught of induced DNA damage causes mutations. These could give rise to predispositions towards some disease states and potentially lead to the development of cancers through the aforementioned damage (de Lemos, 2001, Kirk et al., 2001).

Oestrogens interact in various ways exhibiting protective effects, such as neuro-protection (Latourelle et al., 2010, Agius et al., 2009, DeGiorgio et al., 2002, Bonnefont et al., 1998, Sutcliffe et al., 2008), improved locomotor recovery from spinal cord damage (Mosquera et al., 2014) or protective roles in the development of atherosclerotic plaques (Newnham, 1993). However, oestrogens also have the capacity to generate ROS (Anderson et al., 2003, Di Santi et al., 2014, Yuan et al., 2014, Rettberg et al., 2014, Koong and Watson, 2014). This was shown in some detail involving 84 separate experiments examining sperm and lymphocytes in the Comet assay after treatment with the same oestrogens that were examined in the present study (Anderson, 2005). Catalase and superoxide dismutase were used to abolish the oxygen radical damaging effects showing that ROS can interact with DNA and disrupt the protective functions of cellular antioxidants and repair mechanisms (Anderson, 2005).

There is a relative lack of genotoxicity data examining oestrogenic compounds *in vivo*. Thus in this study, 5 oestrogenic compounds (17β-oestradiol, daidzein, diethylstilboestrol (DES), genistein, and equol) were examined in thirty 12-week old sexually mature male hL rats. The compounds were administered via intra-peritoneal (i.p.) injection. Eight days after exposure (Topham, 1980), various cells were taken from the animals and analysed using the Comet assay.

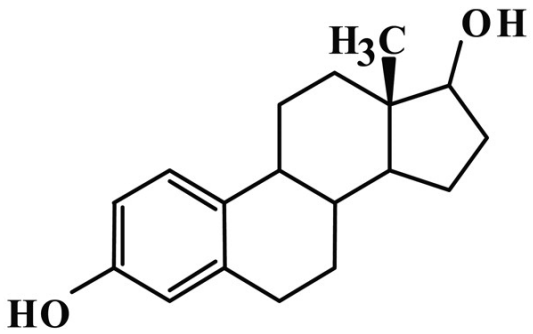
This paradigm is often used in risk assessment research to assess the genotoxic effects of potentially hazardous compounds. Although there are some *in vitro* assays used for studying

germ cells, since there is no suitable system that adequately replicates spermatogenesis *in vitro*, reproductive research in contrast to that in somatic cells, still has a high requirement for *in vivo* assays.

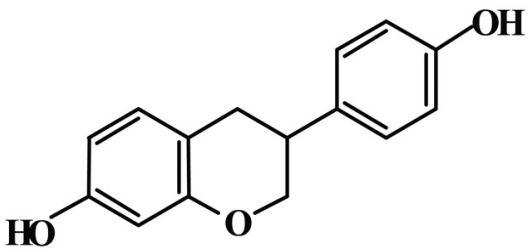
1.1.2 NSAIDs

Nano- technology and nano-therapy have seen enormous growth in recent years. Designing material at this scale or by utilising properties on this scale could lead to more efficacious systems. This is because the altered size of the material can result in a faster mechanism of action as the increased surface-area: volume ratio of nano- compared with bulk-particles can greatly increase reaction rates. Also because of their size, cellular uptake is potentially enhanced, a property that is fully exploited in many cases by coating them in a lipid-soluble coating during their manufacture. If nanotisation of modern compounds and therapeutics has produced a benefit over their current usage, then many of the more long-established drugs (including NSAIDs such as aspirin and ibuprofen) may perhaps find new applications or be effective over broader dose ranges.

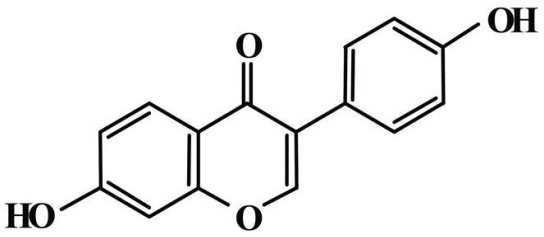
The nano-forms of some agents may have a shift in their toxicity levels. For those nano-particles with enhanced ability to cross lipid-bilayers, it is necessary now also to consider unintended effects on target cells (nano-toxicity). It is therefore important to be aware of negative aspects to the use of nano-materials. Although nano-drugs could play an important part in the future of medicine, appropriate nano-toxicological research to ensure safety and the minimisation of risks to health is necessary.



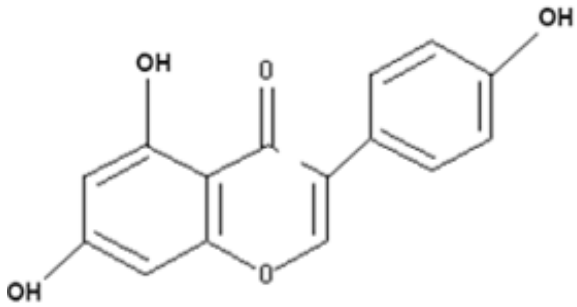
17-beta oestradiol



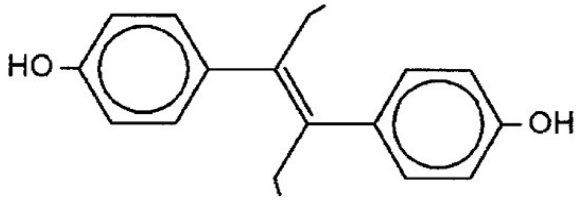
Equol



Genistein



Daidzein



Diethylstilboestrol (DES)

Figure 1.1 An illustration of the chemical structure of the oestrogenic compounds used in this study. Source: Endocr. Pract.©2008 American Association of Clinical Endocrinology and Human Reproduction, Oxford Journal.

1.2 Oestrogenic compounds

It has been reported that oestrogens and their analogues have the potential to cause DNA lesions and show genotoxic effects upon exposure at the cellular level (Anderson et al., 2003, Liehr, 2001, Schallreuter et al., 2006, Toyoizumi et al., 2008). These steroid hormones can cause the development of cancer possibly via free radical generation, an alternative cAMP pathway or by upregualting cellular division (Honkisz and Wojtowicz, 2014, Orostica et al., 2014)

1.3 Cancer and its attributes

Cancer is a multifactorial process, arising from dysfunction of one or more of the key hallmarks of normal cellular function. Diagnosis of cancer and evaluation of its prognosis is carried out by further investigations using scans or invasive exploration, or by measuring the serum levels of tumour markers present (e.g. PSA, CA12-5, CA19-9, CEA or CA15-3). The presence of tumour markers in the blood has also been associated with an increased sensitivity of specific bloods cells to various mutagens, which is a confounding factoring in genotoxicity risk assessment (Malati, 2007).

The methodology for measurement is usually a two-step process firstly using a monoclonal antibody specific to the marker (immunoassay), followed by a chemiluminometric analysis (Gardien et al., 2014). Using this approach a cohort of men with prostate cancer was identified and recruited to evaluate this in the course of the present studies.

1.4 Reactive oxygen species

Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OOH\bullet + H^-$$

Harber-Weiss:

$$O^{\bullet}{}_2^- + H_2O_2 \longrightarrow O_2 + HO\bullet + OH^-$$

The above mentioned reactions describe how the generation of ROS are achieved. The generation of ROS through the Fenton and Harber-Weiss reactions exacerbate the insult to the genome from endogenously produced ROS. Thus, ROS released during mitochondrial respiration are widely generated during in the course of normal metabolism. In addition, some environmental factors, a wide range of chemicals, high oxygen use (e.g. competitive sports) and physiological processes involving autoimmune activation, all also increase the cellular burden of ROS. Such very reactive and unstable molecules may lead to genotoxicity by reacting with DNA, disrupting its structure and potentially its functionality

1.5 Genetic toxicology

Understanding what constitutes genetic toxicology and relating this research to clinical impact is important for preventing various disease states and possibly furthering the understanding of treatments and therapies. Any agent that is able to, or has the potential ability to, inflict genomic insult and thus cause cancerous states, is of great concern. Whether the genomic insult is direct or indirect the ultimate outcome remains the same. These agents may have a threshold, which means that they may only defer genomic insult once a certain

level is achieved or dosage is given. This research is vital for the pharmaceutical industry, government bodies, policy makers, consumers and the general public and is enshrined in law in most countries in the world, in a system of legally enforceable exposure limits based on the latest relevant genetic toxicological findings. As indicated above, the Comet and micronucleus assays are two such assays of importance both for regulatory and fundamental research.

1.6 Comet assay

The Comet assay under alkaline conditions allows measurement of both single-stranded and double-stranded breakages. This method is used through research, academic and as a tool for drug advisory panels on toxicity. It is a reliable, proven and reproducible method that incorporates a semi-automated element to decrease the time taken for analysis. The Comet assay also has a further advantage in that it can be used for a wide range of tissue types and gives information at the individual cell level.

1.7 Micronucleus assay

The statistical power that comes from the micronucleus assay is very considerable. Large numbers can be scored relatively quickly, although identification can occasionally be dependent on the user. The micronucelus assay is one of the methods of choice (as well as the Comet assay), to assess genotoxicity both for fundamental and regulatory research.

The micronucleus assay relies on whole blood samples. If the sample is not collected correctly there is a risk of clots forming and making the sample unusable.

The micronucleus assay shares many of the advantages of the Comet assay in ease of use, reliability and reproducibility, in providing data at the individual cell level and in its applicability to a range of tissues, including blood. A disadvantage of this method however, is

the high background frequency, which could lead to false positive results. The background levels of damage exaggerate genotoxicity. It is therefore important to include appropriate positive and negative control groups. The micronucleus assay, may fail to generate any results if the proper aseptic procedures are not followed. Introduction of infections in the early stages of analysis can occur. Although technically it is labour intensive, is it designed to examine cellular divisions occurring in the blood which no other method can do as effectively.

1.8 Target cells

Oestrogens are principally lipid soluble so potentially can have an effect systemically. It is known that the brain contains oestrogen receptors, however the incidence of brain tumours that could be associated with oestrogens may be low. As some of the compounds administered are synthetic compounds it was important to examine the liver, so see whether the metabolism of these compounds had an effect on the hepatic cells.

Oestrogens are produced in the adrenal cortex, in some peripheral tissues such as adipose tissue and importantly in the testis. This is why the testis were examined, to determine the effect on the germs cells.

In addition, the combined use of blood cells and sperm as a strategy for assessing the genotoxicity of compounds is well established and allows the detection of effects induced in any part of the body (blood cells) as well as potentially heritable effects (sperm). Therefore, it is suitable both for oestrogens and nano-particles.

1.9 Aims & Objectives

1.9.1 Aim

To investigate the genotoxicity of oestrogens and of nanotised or non-nanotised forms of NSAIDs.

1.9.2 Objectives

- Investigate the effects of 5 oestrogenic compounds; 17β-oestradiol and diethylstilboestrol, genistein, equol and daidzein *in vivo* and the effects these have on DNA migration patterns using the Comet assay on hepatic cells, bone marrow cells, blood and testicular cells.
- Assess the genotoxicity of bulk and nano- forms of NSAIDs on blood taken from prostate cancer patients using the Comet assay.
- Assess the toxicological potential of the nano compounds using the micronucleus assay.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and methods

2.1.1 *In vivo* study

2.1.2 Animal procedures

The work was carried out under the project licence from the Pharmacy Department, University of Bradford, and in accordance with ethical procedures and protocols, (Institute of Cancer Therapeutics, University of Bradford, UK).

Experimental Design: 30 male hL rats were used to assess the genotoxic effects on various tissues. The controls received corn oil as the vehicle via i.p. injection and as a diluent for the oestrogenic compounds. The 30 rodents (5 controls and 5 treatment groups each of 5 animals) were killed after 8 days of exposure and cells from the testis, blood, liver and bone marrow were removed and the Comet assay carried out.

2.2 Animals and Husbandry

Sexually mature 12 week old male hooded-Lister rats, (Harlan, Bicester, UK) were chosen because of previous work used on this out-breed species (Sutcliffe et al., 2008, File et al., 2003). The animals were given time to acclimatise before commencing the experiment. Food and water supplies for the animals were available *ad libitum*. They were housed in cages with solid plastic sides and stainless steel grid tops and floors in a room designed for control of temperature (~21°C± 2°) with 45-55% humidity and light cycle (12 hours light/ 12 hours darkness), to mimic *in situ* conditions (Sutcliffe et al., 2008, Anderson et al., 1996).

2.3 Compound Administration

The hL rats were weighed (from 218-244g, with an average weight of 229.12g) and these weights were recorded prior to administration of the oestrogenic compounds and also prior to sacrifice (Table 3.1). They were housed in groups of 5 and tails marked 1-4 with a blank indicating the control. In the Comet assay study, the compounds were administered to the rats with the following doses: (17β-oestradiol [50-28-2], (50 µg/kg/b.wt), daidzein [486-66-8], (250µg/kg/b.wt), DES [56-53-1], (100µg/kg/b.wt), genistein [446-72-0], (250µg/kg/b.wt) and equol [Apin Chemical-531-95-3], (250µg/kg/b.wt). All compounds except equol were obtained from Sigma Chemicals (Latonnelle et al., 2002, Anderson et al., 1996). The doses are based on previous work to establish a dose response curve (Latonnelle et al., 2002, Anderson et al., 1996).

2.4 Comet assay study

The Comet assay was conducted as described in Anderson et al., (1996), (Anderson et al., 2003, Anderson et al., 1996, Singh et al., 1988, Tice et al., 2000). Briefly, an agarose 'sandwich' is prepared on a glass slide with lysed cells between the layers and subjected to electrophoresis. This draws the DNA out of the nuclei forming a 'comet tail' when stained with ethidium bromide and visualised under ultraviolet light. The size and intensity of the tail is therefore proportional to the amount of strand breakage in the genome.

The rats were anesthetised in a CO_2 chamber according to a Schedule I killing method, followed by cervical dislocation. Different cell types were removed after this procedure:

2.4.1 Bone marrow preparation

Cells were removed from the femur after cutting the epiphyses and cells removed by aspiration with PBS (Anderson et al., 1996). Muscle was completely removed from the bone

and using 1 ml of PBS the bone marrow was flushed out. The work was not carried out under sterile conditions, however sterile media and aseptic technique were used to minimise the chances of infection occurring. The bone marrow sample was cleaned thoroughly to ensure no contamination with blood.

2.4.2 Testis preparation

The testes were carefully decapsulated with a fine forceps and tweezers and minced together with the epididymides. The testes were moistened in PBS buffer. A sample of 100µl was taken and mixed together with the second agarose layer. 70µl was added to the pre-coated slides, this was done in duplicate.

2.4.3 Blood preparation

The blood was taken from a cardiac puncture and processed immediately. 100µl of blood were mixed together (1:5) with RPMI- cell culture medium. This was added to 100µl of agarose and embedded on the slide.

2.4.4 Liver preparation

Once the liver had been removed it was then fragmented and cut into small pieces followed by homogenisation of the entire liver. This ensured the cells which were exposure to the oestrogenic compounds would be detected. The liver needed a further step in processing where trypsin was added to the cell suspension (1:5) and this was added to PBS. The trypsin [Gibco], digest removed the cellular matrix and individualised the cells. 100µl of the final cell suspension was removed and added to 100µl of 1% LMP second agarose layer and embedded.

A coding system was used to for the slides and randomly scored. Each slide was given a random letter and slides were chosen randomly.

2.5 Slide staining

60µl ethidium bromide [1239-45-8- Sigma Chemicals], (20µg/ ml) was added to each slide and covered with a cover slip. Care was taken not to penetrate the gel or to contaminate it by touching with the pipette tip.

2.6 Slide scoring

Slide scoring and analysis using the Comet Kinetic Imaging software© 4.0, Liverpool. A microscope linked to a florescence lens was used in conjunction with the Komet4™ software for viewing the comet assay patterns. One hundred nuclei were scored per individual and mean Olive tail moment or %age tail DNA calculated per individual per group. The tail moment is equivalent to the integrated value of density multiplied by migration distance and is considered and recommended as a sensitive and reliable measurement when using various tissue types (Kumaravel and Jha, 2006, Hartmann et al., 2003). The reproducibility and number of cells which can be scored gives the Comet assay statistical power. The parameters were: head threshold 2%, tail threshold 0%, smoothing value 1 (the smoothing value made visualisation of the real data easier by selecting the grey level and the background peak frequency [Anderson, et al., 1996]), background height 20; tail break length 5 (Anderson et al., 1996, Anderson et al., 1998), cells from each slide were analysed using the above software. The slides were decoded after scoring.

2.7 Statistical Analysis

In the Comet assay, the non-parametric Wilcoxon rank-sum test was performed on individual data. The responses from the different cell types were compared with the negative control using the student t-test. Olive tail moment and % tail DNA were examined as both are

recommended (Jha, 2004, Stang and Witte, 2009, Tice et al., 2000). P values <0.05 were regarded as statistically significant.

2.8 *In vitro* prostate cancer study using the micronucleus assay and Comet assay.

2.8.1 Ethical approval

This study was approved by Leeds (Central) Research Ethics Committee (REC reference number: 09/H1313/37) and the Research Support & Governance Office, Bradford Teaching Hospitals NHS Foundation (ReDA number: 1202). Ethical permission was also provided by the University of Bradford Research Ethics Sub-Committee on Research in Human Subjects (reference number: 0405/8).

2.8.2 Whole-blood sample collection:

Blood from prostate cancer patients, suspect prostate cancer patients and healthy volunteers were obtained from the Ethical Tissue Bank, University of Bradford, which has approval from the UK Subcommittee for Research Involving Human Subjects. (Use of these samples was also approved as detailed above by University of Bradford ethical approval: 0405/8.)

2.9 NSAIDs:

Ibuprofen USP was purchased from Albermarle Europe sprl, (Belgium). Pharmcoat 606 (HPMC) was kindly donated by Shinetsu (Japan). Bulk and nano-suspensions of ibuprofen were prepared by Lena Nanoceutics (University of Bradford, UK), (Najafzadeh et al., 2012).

2.9.1 Nano-material methodology

Suspensions of ibuprofen with solid loads of 3% and 4% (w/w) respectively were prepared. The suspending medium consisted of: HPMC (0.5%, w/w), polyvinylpyrrolidone K-30 (0.5%, w/w) and sodium lauryl sulphate (0.1%, w/w) in deionised water (Plakkot et al.,

2011). The milling was carried out using Lena nanoceutics technology DM-100 machine (Sulaiman, 2007).

250 ml of each suspension were milled using 150 ml of 0.2 mm yttrium stabilised zirconium beads (Glen mills, USA). The materials were recycled for 60 min in the milling machine before being discharged in an opaque glass bottle and stored in the refrigerator at (4°) for the duration of the experiments, (Najafzadeh et al., 2012).

2.9.2 Particle size and analysis

The particle size distribution of aspirin and ibuprofen nano-suspensions were determined using the dynamic light scattering technique of the Zetasizer Nano ZS (Malvern instruments, UK). Samples were measured at room temperature using disposable sizing cuvettes. All measurements were carried out in triplicate. The particle size of the stock suspensions were measured in triplicate immediately after milling and then rechecked at the end of the experiments to ensure no significant change in particle size occurred during the various phases of the experiments. The particle size of the bulk powder was measured using the laser diffraction technique (Sympatec Helos, UK). Approximately 20 mg of each drug were transferred into the sample vial and the primary pressure was adjusted to 4 bars while the feeder speed was 40mm/s. Three samples of each drug were measured using R_2 lens (0.25/0.45, 87.5µm).

2.9.3 Zeta potential

The zeta potential for suspensions is the frequency shift depending on the magnitude of the nano-particle size (mV). This was determined using Zetasizer Nano ZS (Malvern instruments, UK). The suspensions were diluted 1:100 using deionised water and measured at 25°C. Clear disposable zeta cells were used. Measurement duration was set as automatic with

a minimum of 10 runs and a maximum of 100 runs. All measurements were made in triplicate, (Najafzadeh et al., 2012). The definition for size of any given nano-medicines set by The National Cancer Institute (NCI), which is: nano-particles are particles with sizes between 1 and 100nm. Nano-particles are composites of therapeutic agents and synthetic carriers of materials, (Najafzadeh et al., 2012).

2.10 The Cytokinesis blocking micronucleus (CBMN) Assay

Micronucleus assay reagents General Laboratory reagents were purchased from Fisher Scientific Co. (Itasca, IL). RPMI 1640 medium (with L-glutamine and 25 mM Hepes), Cytochalasin B (Cat No. C6762) and mitomycin C (Cat No. M0503) were purchased from Sigma-Aldrich (Poole, UK), penicillin-streptomycin solution (Cat No.15140-122) were purchased from Invitrogen Ltd, UK. Slides and cover slips were obtained from VWR international. The Fixogum rubber cement was purchased from (Marabu, Germany). Patient blood was collected into lithium-heparin tubes (vaccuette containers).

2.11 Micronucleus assay protocol:

The samples were cultured and handled within sterile conditions. Patient blood was added to culture flask. One hundred µl of PHA was added to each of the culture flasks containing the 400µl of whole blood and incubated at 37°C in conditions of 5% CO2. At the 44-hour interval, 30µl 1mg/ml Cytochalasin B was added for a final concentration of 6µg/ml. Micronuclei are found in the precursors of erythrocytes in whole blood.

2.11.1 Cell preparation

At the 72 hour time point, the cultures were transferred and centrifuged for 8mins, 800RPM (110xg). The supernatant was removed and cells resuspended. Hypotonic shock was carried out by exposing the cultured cells to 90mM KCl for 15mins at 4°C.

Cultures were then centrifuged and the supernatant removed and cells resuspended. 5ml of Carnoy's solution was added followed by 3 drops of formaldehyde to each culture. A further centrifugation step was followed and, again, the supernatant was removed and the cells were resuspended. This fixation step was repeated twice further. 10µl of cell suspension was applied to microscope slides and then stained using freshly prepared 5% Giemsa in Sorensen buffer, pH 6.8- filtered twice using Whatman 41 filter paper. Once the slides had been rinsed thoroughly, and left to dry (preferably over night), cover slides were mounted onto the slides using Fixogum rubber cement.

2.12 Cell scoring

Using bright-field light microscopy at 1000 x magnification, slides were scored using criteria, as recommended by Fenech *et al.,* (2003; 2007). MNi were scored each from binucleated (BiNC) and mononucleated (MonoNC) cells. The nuclear division index (NDI) was used as an indicator of the cytotoxicity and the following calculation was used to find the NDI: NDI = (M1 + 2(M2) + 3 (M3) /N

Where: M1 = mononucleated cells, M2 = binucleated cells, M3 = multinucleated cells, N = the total number of viable cells scored. The number of cells scored for the investigation was 1000 cells /person/ treatment group.

CHAPTER 3

IN VIVO STUDY USING THE COMET ASSAY

3.1 Introduction

There is a lack of genotoxicity data examining oestrogenic compounds *in vivo*. Thus in this study, 5 oestrogenic compounds (17β-oestradiol, daidzein, diethylstilboestrol (DES), genistein, and equol) were examined in thirty 12-week old sexually mature male hL rats. The compounds were administered via i.p. injection, as this method of administration has been used before (Rakhila et al., 2014, Pinto et al., 2006). Eight days after exposure, various cells were taken from the animals and analysed using the Comet assay. Cells were examined at this time point because previous studies have shown genetic effects on both sperm and somatic cells eight days after exposure, (Anderson et al., 1996, Topham, 1980b).

3.1.1 Materials and methods.

The methods used in this chapter have been described in chapter 2.

3.2 Results

Table 1 shows the weights of the rats prior to administration of the compounds and before sacrifice. There were no differences in the weights of the animals after dosing and before sacrifice, except in the group dosed with genistein, showing that compounds have no adverse effects.

Table 3.1 Rat weights (gms) at dosing and prior to killing on day 8. *P* values compared to the vehicle control group (student's *t*-test).

Corn oil	**Rat weight at dose (gms)**	**weight prior to killing (gms)**
Vehicle Control	218	284
Vehicle Control	230	297
Vehicle Control	232	296
Vehicle Control	229	288
Vehicle Control	244	312
Mean wt. (gms)	**230.6**	**295.4**
Dose 50mg/kg/b.wt.		
17β-oestradiol	219	285
17β-oestradiol	231	290
17β-oestradiol	230	290
17β-oestradiol	215	288
17β-oestradiol	231	266
Mean wt. (gms)	**225.2**	**283.8**
p=values	***p*=0.34**	**p=0.12**
Dose 100mg/kg/b.wt.		
DES	228	298
DES	221	270
DES	239	302
DES	232	300
DES	228	269
Mean wt. (gms)	**229.6**	**287.8**
p=values	**p=0.85**	**p=0.42**
Dose 250mg//kg/b.wt.		
Genistein	232	307
Genistein	226	390
Genistein	244	314
Genistein	233	285
Genistein	239	281
Mean wt. (gms)	**234.8**	**315.4**
p=values	**p=0.44**	**p=0.35**
Dose 250mg//kg/b.wt.		
Daidzein	219	298
Daidzein	223	284
Daidzein	225	287
Daidzein	224	303
Daidzein	230	300
Mean wt. (gms)	**224.2**	**294.4**
p=values	**p=0.19**	**p=0.87**
Dose 250mg//kg/b.wt.		
Equol	227	290
Equol	228	294
Equol	231	291
Equol	235	312
Equol	230	274
Mean wt. (gms)	**230.2**	**292.2**
p=values	**p=0.93**	**p=0.69**

3.3 The Comet assay

Figures 3.1 and 3.2 show images of comets in lymphocytes and sperm. Viewing magnifications are given for all figures.

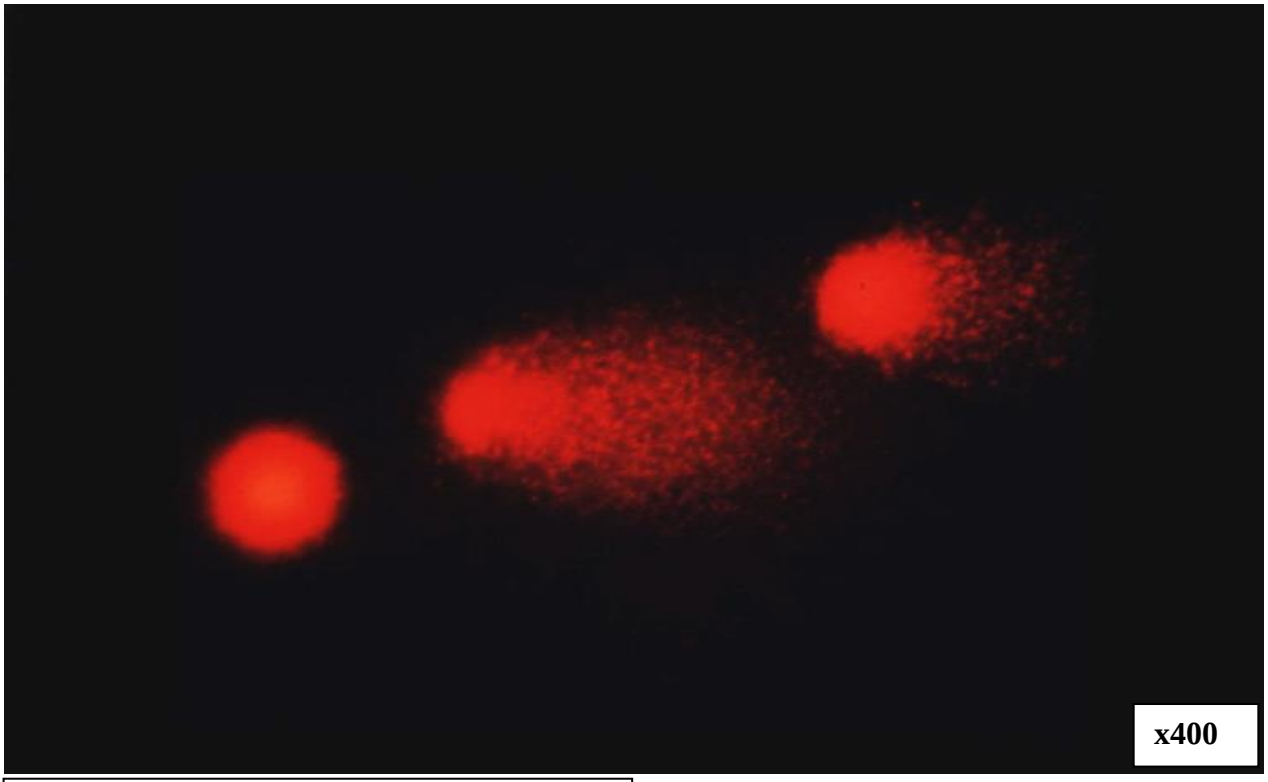


Figure 3.1 Comet images of lymphocytes

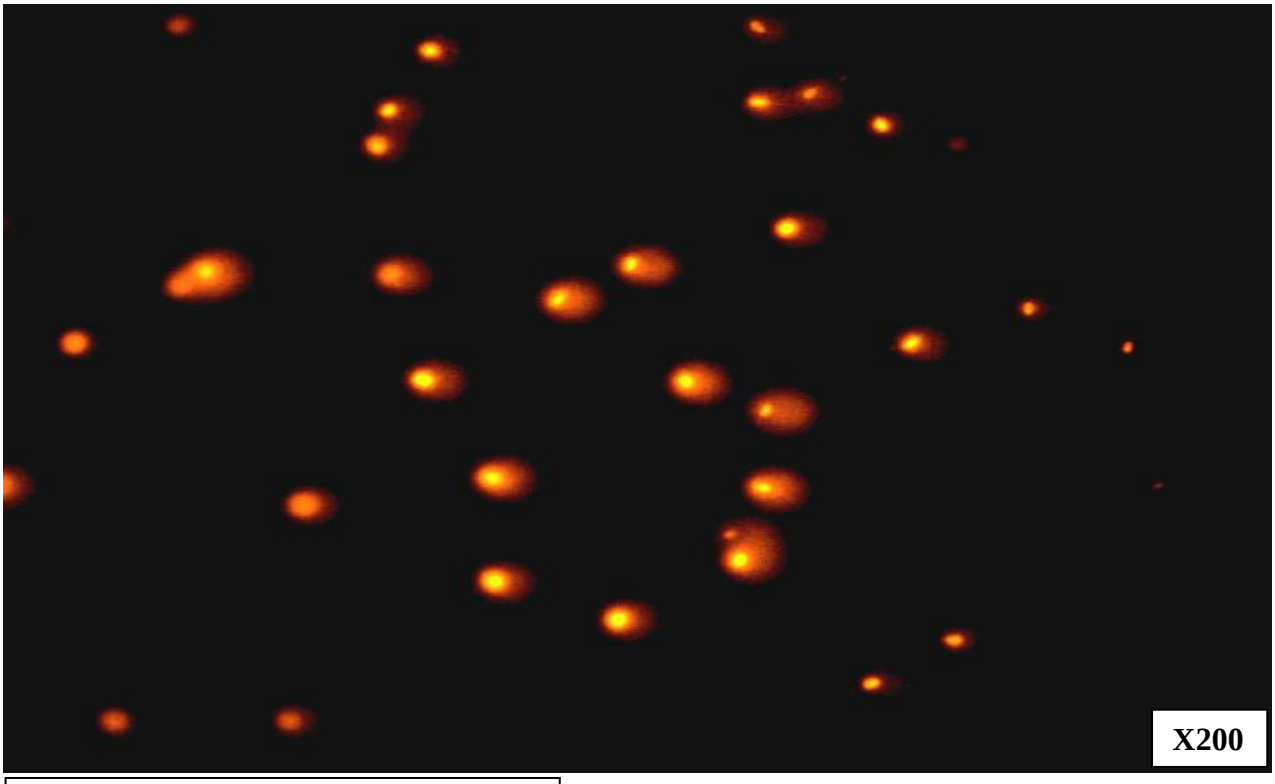


Figure 3.2 Comet images of sperm.

Figures 3.3-3.6 show the Olive tail moment and % tail DNA for cells from the liver, testis, blood and bone marrow respectively. Whilst there were some variations in the values with the different oestrogens, the only two values that have statistical significance by comparison with the negative control were testicular cells treated with daidzein for % tail DNA ($p= 0.043$) [Figure 3.4] and similarly blood cells treated with DES ($p= 0.034$) [Figure 3.5].

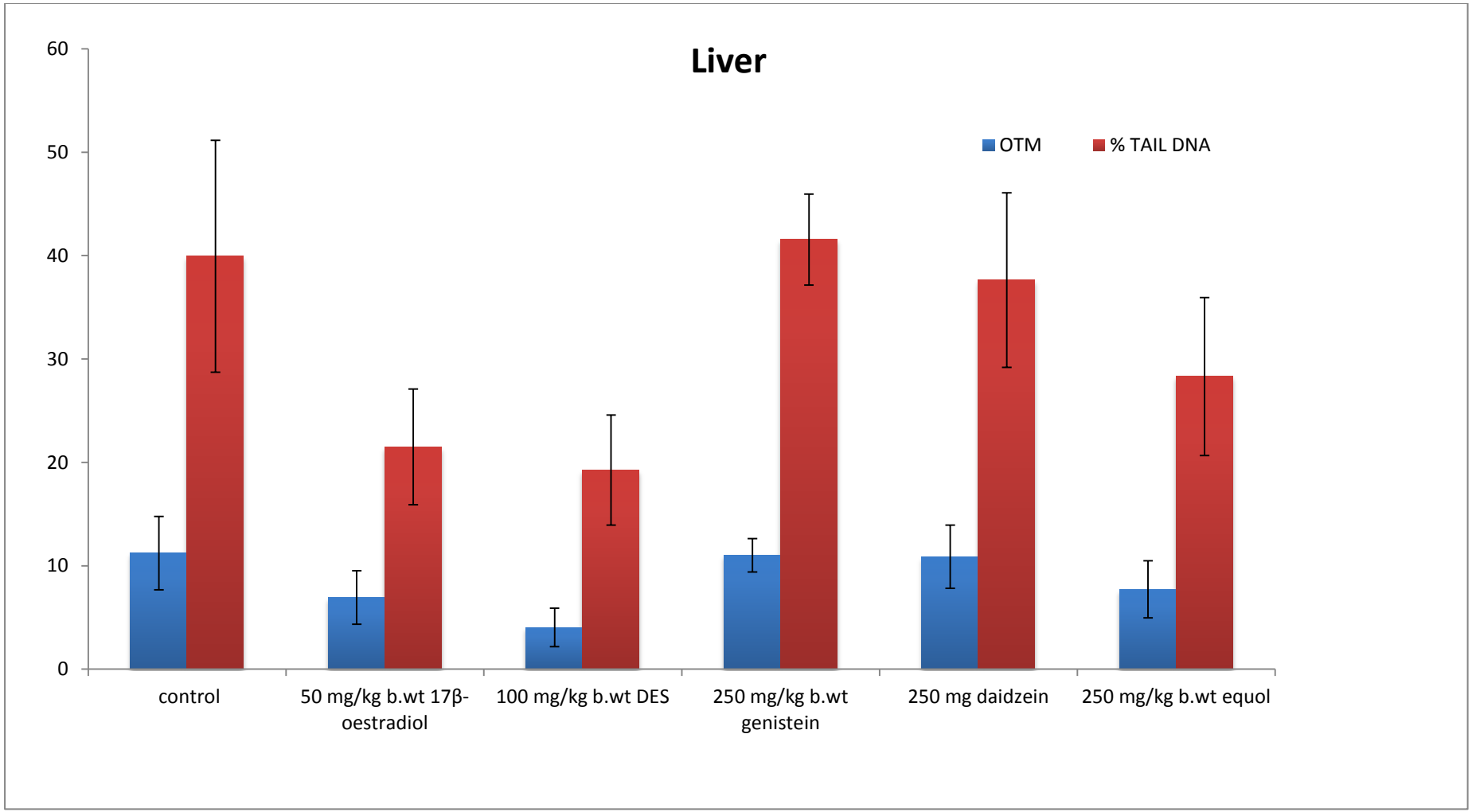


Figure 3.3. The Olive tail moment and % tail DNA for cells from the liver. Data are expressed as mean ±SEM

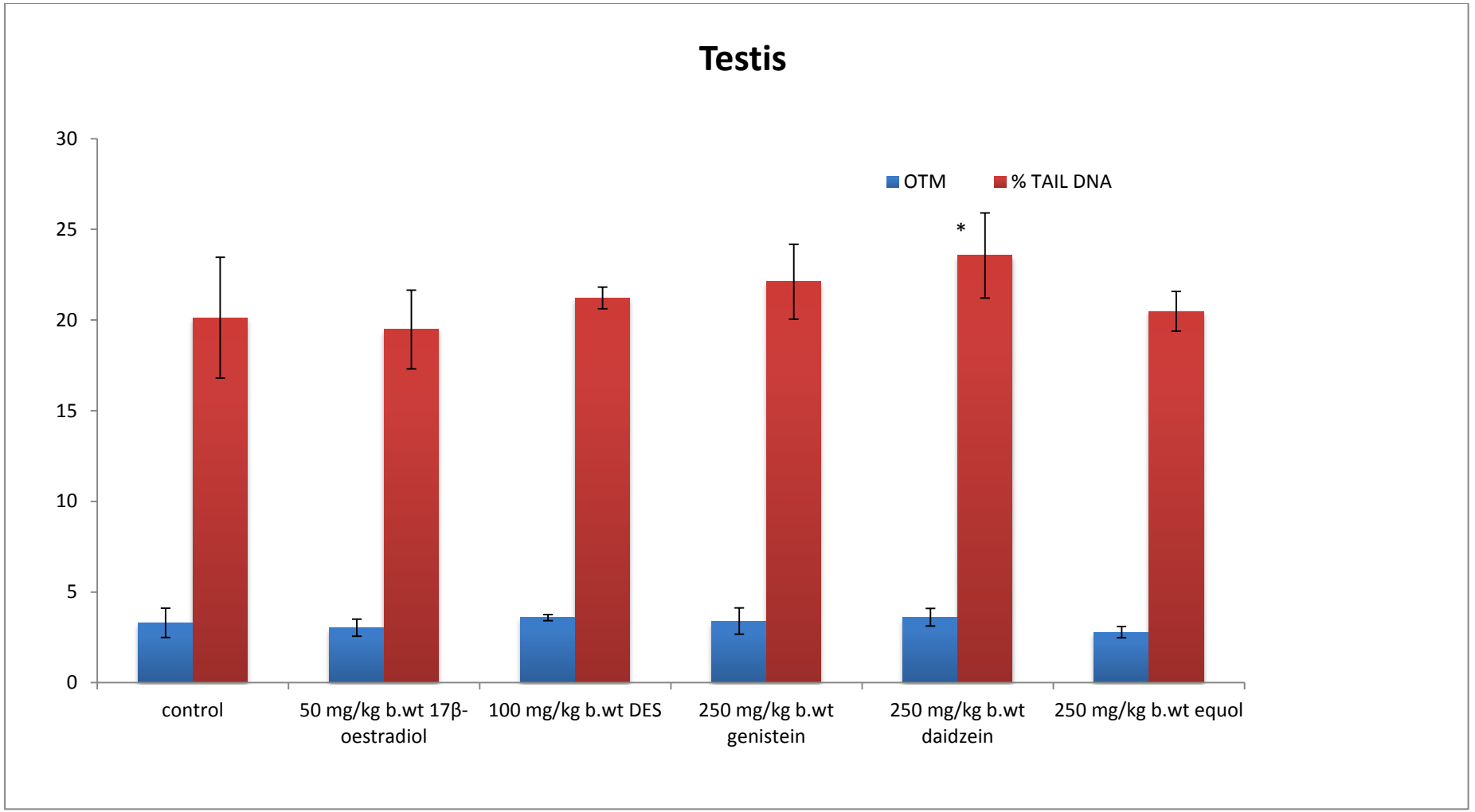


Figure 3.4. The Olive tail moment and % tail DNA for cells from the testes *significance by comparison with the negative control (p=0.043). Data are expressed as mean ±SEM

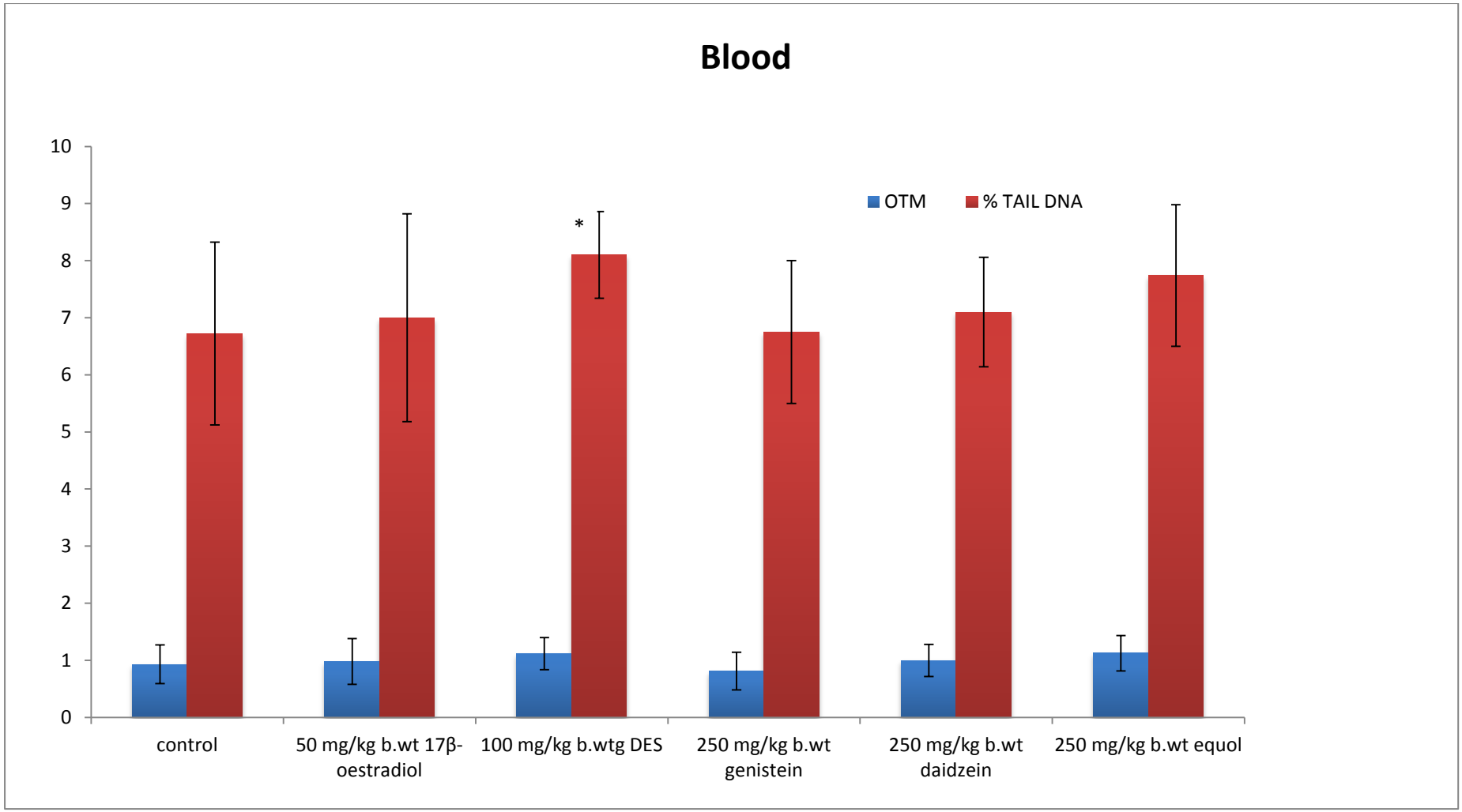


Figure 3.5. The Olive tail moment and % tail DNA for cells from the blood. *significance by comparison with the negative control (p=0.034). Data are expressed as mean ±SEM

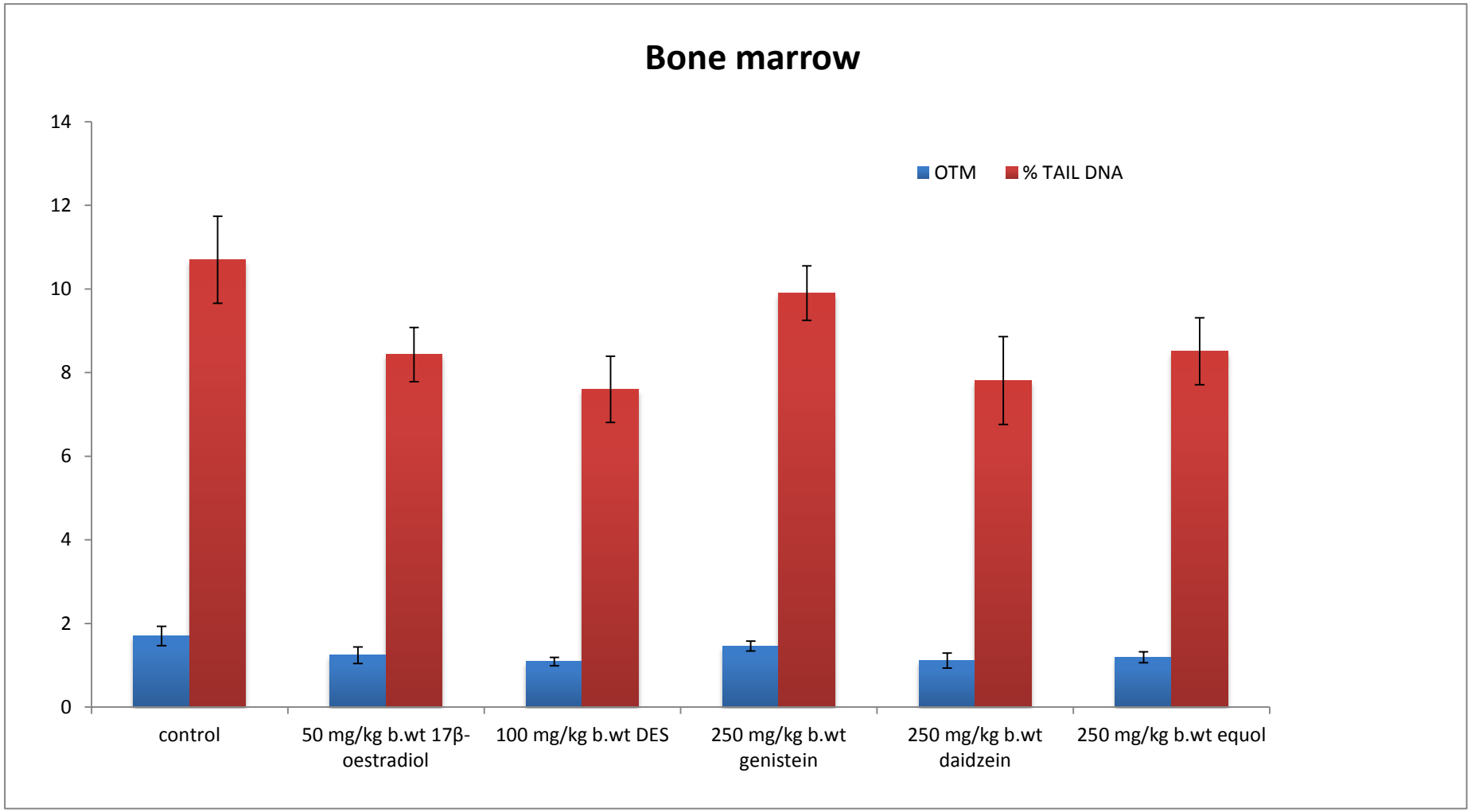


Figure 3.6. The Olive tail moment and % tail DNA for cells from the bone marrow. Data are expressed as mean ±SEM

3.4 Discussion

This study was designed to investigate the effects of 5 oestrogenic compounds *in vivo* to determine whether DNA damage occurred and if so to what extent. The five oestrogens were chosen because all have been shown to produce oxidative stress in a total of 84 experiments *in vitro* in the Comet assay both in lymphocytes and sperm. This stress can be abolished in the presence of catalase and to a lesser extent with superoxide dismutase, as discussed earlier (Anderson et al., 2003), which indicated that DNA damage could possibly occur *in vivo*.

The study did not use a positive control group that could lead to DNA effects in the tissues of interest because there is not one available for the four tissues investigated. However, the 5 oestrogenic compounds have previously all shown to be positive in sperm and lymphocytes, so the timing of the response determination was based on that for bleomycin operating through an oxidative stress mechanism previously detected *in vivo* (Anderson et al., 1996). It is suggested that bleomycin works by chelating metal ions, producing a component which reacts with oxygen and this produces free radicals. Also DES produced a response in post meiotic germ cells (sperm head abnormalities), after a single i.p. dose (Topham, 1980a). It is known that alkylating agents like ethyl and methyl methane sulfonate are positive in post-meiotic germ cells after a single dose and chemicals like the antimetabolites methotrexate and mercaptopurine are positive in meiotic germ cells and ethyl and methyl nitosourea are positive in pre-meiotic germ cells. DES falls into the first category.

It is recognised that there are statistically significant effects for one Comet parameter; % tail DNA but not for Olive tail moment. These results are relatively minor and therefore require confirmation by further investigation. The Olive tail moment is suggested to be an appropriate index of induced DNA damage in considering both the migrations of the genetic material as well as the relative amount of DNA in the tail. Another dose to show toxicity to

the target tissue has to be investigated to confirm negative results. Further work could investigate this.

It is well known that oestrogens can produce hazardous effects in humans by disrupting the spermatogenic cycle by impairing spermatogenesis (Nakazumi et al., 1996).

The criteria used to choose the tissues used in this study were to include testicular cells corresponding to sperm, and blood, corresponding to lymphocytes, of an earlier *in vitro* study (Anderson et al., 2003). The reasoning behind the choice of dose was based on a conversion of the concentrations /doses of our *in vitro* study from µg/ml to mg /kg body weight (equating 1ml of medium *in vitro* to 1g body weight *in vivo*) and intra-peritoneal injection. This was the most common route of exposure for the single dose dominant lethal studies (Anderson et al., 1996) in male rodents and other effects on sperm (Topham, 1980a), on which this study was based.

There are 2 ways to carry out a dominant lethal assay in males treated once i.p. The original method in which male animals are mated for eight weeks (mice) or ten weeks (rats) to virgin female mice over the spermatogenic cycle and mated once a week or every few days to determine at which stage of the spermatogenic cycle the chemical is at its most active. There is a second method where animals are mated after 2 doses, one non-toxic and one toxic. The animals are mated for 8-10 weeks and sampled twice. The toxic dose will ensure that the animal received the compound when a negative response is seen.

Oestrogenic compounds play an antioxidant and pro-oxidant role in normal cellular homeostasis. e.g. it is known that oestrogens have a neuro-protective role in the brain, but can also be involved in the development of atherosclerosis, which involves oxygen radical damage (Newnham, 1993).

The generation of ROS occurs through every day normal metabolism and metabolic events. Reactive oxygen species exist due to a loss or gain of an electron from a non-radical species. During the *de novo* synthesis of ROS, mechanisms exist *in vivo* to limit the amount of damage. The GG-NER and BER mechanisms repair DNA lesions while actions of antioxidants such as catalase, superoxide dismutase and glutathione peroxidase, convert the radicals into non reactive species (Anderson et al., 1996, Schallreuter et al., 2006). It is understood that the generation of ROS through the Fenton and Harber-Weiss (Schallreuter et al., 2006) reactions help perpetuate the assault on the genome. Crucial to these reactions are the unstable species that remove electrons from other substrates to help stabilise the structure of the oxygen molecules

Ultimately, alterations produced this way will cause cellular dysfunction and promote tumour development mediated through the generation of ROS damaging the DNA (Anderson et al., 2003).

In this study weights did not differ significantly from the vehicle control, suggesting there were unlikely to be adverse effects due to weight changes. Imply by recording the weights of the rats can give you an indication to onset of pathogenesis. Genistein showed a slight increase in weight. This may have been due to the fact that is it a phytoestrogen, but daidzein which is also a phytoestrogen showed no weight gain. Daidzein and DES produced responses of borderline statistical significance in % tail DNA in the testis and blood. A longer duration after exposure to the oestrogenic compounds may cause further or less DNA damage to occur, depending on normal cellular repair mechanisms. This was not the case for the testis, where the damage was measured in the late spermatid stage of the spermatogenic cycle, where it was most likely to occur. For another agent, bleomycin, which involves an oxygen radical mechanism, the effect was examined at a similar stage after treatment, as discussed earlier (Anderson et al., 1996). The oestrogens were also given as a single acute

dose in this study. This may have affected the bioavailability of the oestrogens, but to what extent is unknown.

Oestrogens can be converted to catechol oestrogen-3,4-quinones and oestradiol-3,4-quinone becoming electrophilic metabolites, which react with DNA forming depurinating adducts (Cavalieri and Rogan, 2006). Oxidation of catechols to semiquinones and quinones is a mechanism of tumour initiation. The same can be said for synthetic oestrogens such as DES. It is known that some oestrogens e.g. genistein is an isoflavone found in many different types of soy beans and meats and has the ability to act as an antagonist to oestradiol without creating the genomic effects because it is unable to recruit necessary co-transcriptional factors due to its conformation (de Lemos, 2001, Merritt and Jenks, 2004).

In conclusion, in male hL rats, i.p. administration of the oestrogenic compounds produced borderline statistically significant increases in DNA damage seen *in vivo* in the testis with daidzein and blood with DES.

CHAPTER 4

THE GENOTOXIC EFFECTS OF NANO- AND BULK FORMS OF NSAIDS ON BLOOD SAMPLES FROM PROSTATE CANCER PATIENTS

4.1 Introduction

Biomedical sciences research endeavour to determine new and innovative approaches to treat patients. The development of nano-materials and particles bring the possibility of a more direct approach to treatment limiting unwanted side effects. Developments of nanotised therapeutic drugs are fast approaching the clinical trial phases. These new forms of therapeutic drugs and materials need to stand up to the rigours and scrutiny of patient safety, risk assessments and toxicological screens. Therefore we must further our understanding of the safety of these materials as well as the mechanisms of action.

Data from epidemiological studies carried out in 2012, indicate that over 25% of all cancers are related to chronic or unresolved inflammation (Vendramini-Costa and Carvalho, 2012). A prime example of this is seen in patients who are diagnosed with mesothelioma. The chronic inflammatory response is initiated by the presence of asbestos fibres that the immune system is incapable of removing. The accumulation of macrophages release tumour necrosis factor-α (TNF-α) and NF-kB. These pro-inflammatory proteins promote the use and exposure of ROS and other potential mutagens to the mesothelial cells.

4.2 Prostate cancer

This part of the study investigated the effects of nanotised forms of NSAIDs. The study used blood taken from patients with known or suspected prostate cancer and looked at the effects, comparatively, against the nano and bulk forms of the NSAIDs.

Prostate cancer is thought to be the second highest cause of cancer deaths in men. Androgens potentiate and sustain abnormal cellular growth of the prostate gland. Anti-androgen treatment is effective and is the recommended treatment administered. Epithelial cells of the prostate gland produce a tumour marker which is used to assess both the prognosis and

diagnosis of prostate cancer. An increase in prostate specific-antigen (PSA) are associated with benign prostatic hyperplasia, enlargement of prostate gland, and changes in the transitional zone in the urethra are seen.

In Europe, it was estimated that 417,000 new cases of prostate cancers were diagnosed of which 92,300 would have died. Worldwide more than 1.11million men were estimated to have been diagnosed in 2012, of which 307,000 fatalities were estimated (Cancer Research UK 2012).

4.3 Detection and measurement of PSA

The PSA method is used in conjunction with digital rectal examination to aid the detection of prostate cancer. PSA is detected in males with normal, benign hypertrophic and malignant prostate tissue. One commonly used method for measurement by many NHS trusts in the UK uses a Siemens Advia Centaur. The PSA assay is a two-site sandwich immunoassay with direct chemiluminescence technology, which uses constant amounts of two antibodies. The first antibody, in the 'lite-reagent' phase, is a polyclonal goat anti-PSA antibody labelled with acridinium ester. The second antibody, in the solid phase, is a monoclonal mouse anti-PSA antibody, which is covalently coupled to paramagnetic particles.

Solid phase and lite reagent are added to the sample; separation, aspiration and washing take place, then acid and base reagent are added to initiate the chemiluminescent reaction. A direct relationship exists between the amount of PSA present in the patient sample and the amount of relative light units detected by the analyser

Reference Interval	Male: Up to 49 years old: <2 ug/L. 50 – 59 years old: <3 ug/L. 60 – 69 years old: <4 ug/L. Greater than 69 years old<5 ug/L.

Table 4.1.Guidelines from the Royal College of Pathology- PSA levels.

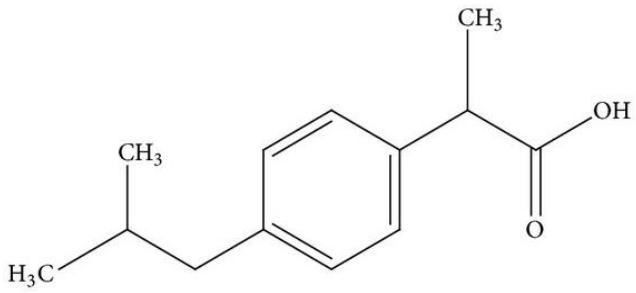
4.4 Material and methods

These are detailed in Chapter 2.

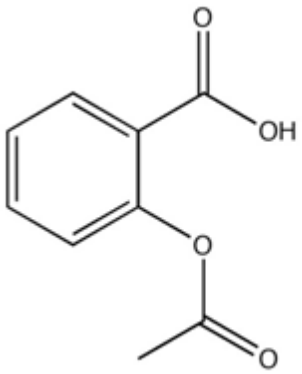
4.5 Experimental Aim

Our experimental aim is to see whether the effects of nanotised NSAIDs compared to their bulk forms show an increase or decrease in micronuclei. We know from previous work carried out from our lab (Anderson et al., 2014), that patients with diagnosed cancer, have sensitised lymphocytes and are more prone to genomic insult. Thus we wish to determine whether sensitised lymphocytes will exhibit a higher or lower numbers of micronuclei, indicating the toxicity of the nanotised drugs, and whether this be can assist in assessing the clinical use for nanotised NSAIDs.

The drugs to be investigated are aspirin and ibuprofen as show in figure 4.1-4.2



The chemical structure of ibuprofen



The chemical structure of aspirin (acetylsalicylic acid)

Figure 4.1 Showing the structural formulae of ibuprofen and aspirin

4.6 Results

The size for the nano particles used in the present study are shown below. (These data have been generated by Dr M. Isreb, School of Pharmacy, University of Bradford). They are in the desired range.

Suspension name	Time of measurement	Average particle size (nm)	Polydispersity index (arbitrary units)	Zeta potential value (mV)
Ibuprofen nano-suspension 3%	Before cells treatment	323±6.4	0.2± 0.01	-2.1
	After cells treatment	340±1.2	0.3±0.001	
Aspirin nano-suspension 4%	Before cells treatment	289±3	0.3±0.03	-6.1
	After cells treatment	299±6.3	0.3 ± 0.05	

Table 4.2 Average particle size, polydisersity index and zeta potential values of the nano-suspensions. (n=3).

Suspension name	Average particle size (**µm**)	Volume Mean Diameter(**µm**)
Ibuprofen	52.80 ± 4.37	20.50
Aspirin	78.30 ± 0.23	44.57

Table 4.3: Average particle size (x90) and the volume mean diameter of the bulk powder (as received) of aspirin and ibuprofen (n=3).

The micronucleus assay data is shown in table 4.4. Blood taken from two prostate cancer patients were investigated at 3 doses. The highest dose was toxic to the cells. Only ibuprofen data are shown, as the nano particles of aspirin conglomerated. For ibuprofen the bulk version produced more micronuclei than nanotised form. In both patients showed similar results for binucleated and mononucleated cells. Table 4.5 shows the NDI and values are within the acceptable range

In figure 4.2, Olive tail moments were compared in the nano and bulk versions of aspirin and ibuprofen in controls, and in suspected and diagnosed prostate cancer patients. In general, the nano version produced lower responses than the bulk versions but statistical significance was not achieved.

The data contributing to figure 4.2 are presented in Table 4.6, confirming that in general, the nanotised forms of the drugs showed lower levels of damage than the bulk forms or the controls. Furthermore, in the prostate cancer patients and controls, levels of damage were highest with the bulk forms. However, statistical significance was not achieved.

Patient 1															
Treatment	**Mono 1**	**Mono 2**	**Mono 3**	**Mono>3**	**Bi Total**	**BiMn1**	**BiMn2**	**BiMn3**	**BiMn>3**	**BiBud1**	**BiBud2**	**BiNPS1**	**MultiMn1**	**MultiMn2**	**MultiMn3**
Neg Control	0	0	0	0	982	17	0	0	1	0	0	0	0	0	0
Ibu B 250mg	15	0	1	0	925	61	3	1	2	7	0	21	0	0	0
Ibu N 250mg	21	3	0	0	965	44	2	2	0	10	0	33	1	0	0
Ibu B 500mg	53	4	0	0	903	91	6	1	0	28	1	27	0	0	0
Ibu N 500mg	25	2	0	0	975	34	1	0	1	19	4	33	0	2	0
Ibu B 1000mg	###	###	###	###	###	###	###	###	###	###	###	###	###	###	###
Ibu N 1000mg	###	###	###	###	###	###	###	###	###	###	###	###	###	###	###
Patient 2															
Treatment	**Mono 1**	**Mono 2**	**Mono 3**	**Mono>3**	**Bi Total**	**BiMn1**	**BiMn2**	**BiMn3**	**BiMn>3**	**BiBud1**	**BiBud2**	**BiNPS1**	**MultiMn1**	**MultiMn2**	**MultiMn3**
Neg Control	1	0	0	0	971	29	0	0	0	0	0	3	4	0	0
Ibu B 250mg	16	3	0	0	914	79	6	0	0	2	0	23	12	1	0
Ibu N 250mg	9	0	0	0	946	51	3	0	0	0	0	3	16	2	1
Ibu B 500mg	4	0	0	0	944	56	0	0	0	0	0	8	0	0	0
Ibu N 500mg	3	0	0	0	961	35	4	0	0	1	0	1	4	1	0
Ibu B 1000mg	###	###	###	###	###	###	###	###	###	###	###	###	###	###	###
Ibu N 1000mg	###	###	###	###	###	###	###	###	###	###	###	###	###	###	###
###= Cells obliterated															

Table 4.4. The scored micronuclei comparing the bulk and nanotised versions. Due to conglomeration of nanoparticles used, results from aspirin analysis are not shown.

Table Legend:

Mono1	Mononucleated cell with 1 micronuclei	BiMn3	Binucleated cell with 3 micronuclei
Mono2	Mononucleated cell with 2 micronuclei	BiMn>3	Binucleated cell with >3 micronuclei
Mono3	Mononucleated cell with 3 micronuclei	BiBud1	Binucleated cell with 1 nuclear bud formation
Mono>3	Mononucleated cell with >3 micronuclei	BiBud2	Binucleated cell with 2 nuclear bud formation
BiTotal	Total number of binucleated cells	BiNPS1	Binucleated cell with nucleoplasmic bridge
BiMn1	Binucleated cell with 1 micronuclei	MultiMn1	Multinucleated cell with 1 micronuclei
BiMn2	Binucleated cell with 2 micronuclei	MultiMn2	Multinucleated cell with 2 micronuclei

Drug	Dose	Form	Group	NDI	Drug	Dose	Form	Group	NDI
IBU	250	Bulk	1	1.84	IBU	250	Bulk	2	1.83
IBU	250	Nano	1	1.87	IBU	250	Nano	2	1.90
IBU	500	Bulk	1	1.72	IBU	500	Bulk	2	1.89
IBU	500	Nano	1	1.87	IBU	500	Nano	2	1.92

Table 4.5. The scored micronuclei comparing the bulk and nanotised versions. Due to conglomeration of nano-particles used, results from aspirin analysis are not shown.

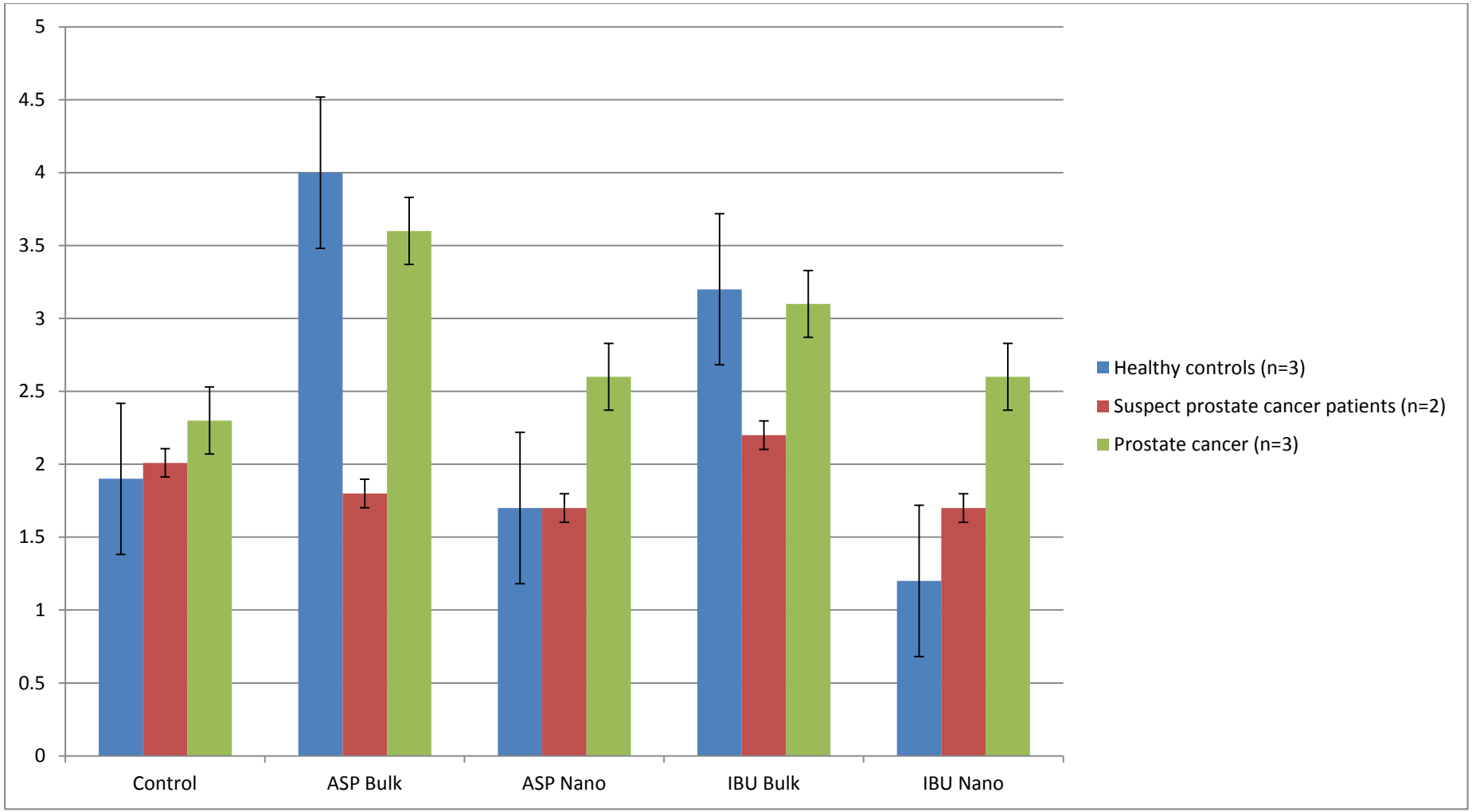


Figure 4.2. After a dose response was established, the nano NSAIDs were compared to their bulk versions using the OTM. Clastogenicity of the bulk and nano forms of the compounds are compared.

	Control	ASP Bulk	ASP Nano	IBU Bulk	IBU Nano
Healthy controls (n=3)	1.85	3.95	1.74	3.24	1.21
Suspect prostate cancer patients (n=2)	2.01	1.85	1.68	2.16	1.77
*Prostate cancer (n=3) excluding outlier	2.32	3.6	2.64	3.14	2.66
**Prostate cancer (n=3) including outlier				3.14	3.71

Test group	Code					
Prostate Cancer	5072PC	2.89	4.51	2.67	4.30	5.78
	5169PC	1.80	2.73	2.11	2.08	2.64
	5329PC	2.28	3.57	3.14	3.04	2.70
	Mean	**2.32**	**3.60**	**2.64**	**3.14**	**3.71**
Suspect Prostate Cancer	5081SPC	2.12	1.98	1.97	2.07	1.99
	5082SPC	1.91	1.71	1.39	2.25	1.54
	Mean	**2.01**	**1.85**	**1.68**	**2.16**	**1.77**
Controls	37cont	1.77	7.77	2.39	6.90	1.37
	J12-6-13 cont	2.00	2.55	1.72	1.19	1.11
	O12-6-13 cont	1.79	1.54	1.10	1.65	1.14
	Mean	**1.85**	**3.95**	**1.74**	**3.24**	**1.21**

Table 4.6. Raw data generated from the Comet assay.

4.7 Discussion

In this study, DNA damage in peripheral lymphocytes blood samples of individuals with suspected and diagnosed prostate cancer have been compared to healthy controls. Treatments with either the bulk (suspension was prepared from the powders as received) and nano-drug versions of aspirin and ibuprofen in the micronucleus assay using the method of Fenech (2007).

Chronic inflammation is now known to contribute to several forms of human cancer, with an estimated 20% of adult cancers attributable to chronic inflammatory conditions caused by infectious agents, chronic non-infectious inflammatory diseases and/or other environmental factors. There is evidence for a role for chronic inflammation in prostate cancer aetiology, with a specific focus on recent advances regarding the following: (i) potential stimuli for prostatic inflammation; (ii) prostate cancer immunobiology; (iii) inflammatory pathways and cytokines in prostate cancer risk and development; (iv) proliferative inflammatory atrophy (PIA) as a risk factor lesion to prostate cancer development; and (v) the role of nutritional or other anti-inflammatory compounds in reducing prostate cancer risk (Sfanos and De Marzo, 2012).

There is some speculation that infection in the prostate gland may be responsible for the chronic immune response. This chronic inflammation could be the main factor initiated in the release of ROS in the microenvironment, thus giving the rise to cancer. In 2009 a study at Queen's University, Belfast, investigated whether *Propionibacterium acne* may be a cause for prostate cancer by causing a chronic inflammatory response.

The evidence from our work suggests that there is a reduction in the number of micronuclei formed when exposed to the nanotised version and compared with their bulk counterparts. As the nano-particles could enter the cells more readily, they could possibly forego some of the

interactions that usually occur when administering drugs. Our studies seems to validate other studies we have carried out in our laboratory on nano-toxicology; however the link between prostate cancer, ROS, nanotised NSAIDs and toxicology is relatively undiscovered. We have found that there is a decrease in MN formation. Similar effects of NSAIDs on cancer have been described by Park, et al (2014). This could be explained by possible direct beneficial effect on cancers and tumour biology (Park et al., 2014). This can been further seen from the down-regulation of the inflammatory response from within the tumour microenvironment (Hussain et al., 2012).

The methods used in these investigations were chosen because these methods are described in the OCED guidelines on carrying out toxicological studies. The reproducibility, effectiveness and statistical power means that these methods can incorporate a wide range of different sized studies whilst be reliable in its accuracy and precision. Although the Comet assay is semi-automated, there is still the element of background inferences which can cause bias. Likewise, with manual scoring of the micronuclei. The clear definition of a micronuclei can be different from one researcher to another. Although the Comet assay is more susceptible to background noise, current methods in combination with software improvements mean that interferences are reduced.

The data generated from the Comet assay, although the n numbers in each group were small and statistical significance was perhaps therefore not achieved, demonstrate a clear reduction in the levels of genotoxicity of the nano-forms, although the suspected prostate cancer patient did not follow this trend. This may have been because these patients do not necessarily have prostate cancer and maybe suffering from other pathologies and have other, unknown, treatments. This reduction in toxicity is interesting and potentially important as it suggests that possibly increasing the reactivity of these drugs by nanotising the particles might not increase toxic interactions. Nano-NSAIDs may thus be not only more effective but also safer.

CHAPTER 5

DISCUSSION

5.1 General discussion

The extent of genotoxicity of 5 oestrogens *in vivo* in male hL rats, as well as the effects of nanotised non-steroidal anti-inflammatory drugs on prostate cancer patients have been examined. Potentially, both may involve reactive oxygen species at the centre of their mutagenicity. Technologies evolve to become more efficient and financially viable, as in the case of nano-technology, but toxicology must keep pace with this evolution. As with many *de novo* pathways and mechanisms within the body, defects and disruptions and can lead to an unregulated and uncontrolled response as seen in cancer. The effects of ROS and their unwanted responses have had much scientific scrutiny and investigations because of their association with cancer development. It is believed that ROS play a crucial role in the generation of cancer.

In this *in vivo* study, responses were seen with daidzein in testis and DES in blood. Oestrogens can be converted to catechol oestrogen-3,4-quinones and oestradiol-3,4-quinone becoming electrophilic metabolites, which react with DNA forming depurinating adducts (Cavalieri and Rogan, 2006). Oxidation of catechols to semiquinones and quinones is a mechanism of tumour initiation. The same can be said for synthetic oestrogens such as DES.

NSAIDs represent a critically important class of medications useful in numerous musculoskeletal and inflammatory diseases which also involve oxygen radical damage. As NSAIDs may regulate NAG-1 protein expression, this may mediate induction of ROS by initiating the associated intrinsic pathways (Vaish et al., 2013). This significant enhancement of intracellular ROS in turn increases apoptosis.

There is increasing evidence for the preventative activity of NSAIDs in prostate cancer. Zhang et al. (2010), showed the suppression of cellular growth by G1 arrest at low concentrations (<10 μmol/l); and at higher concentrations (≥10 μmol/l) suppression was

associated with caspase-mediated apoptosis. Shebl et al. (2012), analysed patient data from 29,450 men aged between 55-74 in relation to prostate cancer risk. Daily aspirin use, but not ibuprofen, reduced prostate cancer risk. It is though that the inhibition of COX and cell proliferation in combination with induced apoptosis is responsible. Similarity in a study by Dhillon et al. (2011), showed a 10% lower risk of prostate cancer incidence in patients taking two adult-strength aspirin tablets weekly. Again it is though that the combination of inhibition of cell proliferation and COX; and induction of apoptosis was responsible.

Salinas et al. (2010), showed a reduction in prostate cancer risk by 21% in aspirin users compared with non-users. This study identified the involvement of PTGS enzymes and inhibition of cell proliferation.

There also seems to be evidence that a relationship exists between NSAIDs and the generation of ROS in the micronucleus assay (Kim and Yu, 2014), as found in the present study in the micronucleus and the Comet assay. This work seems to support the suggestion that NSAIDs on a nano scale could be advantageous for patients and thus influence future therapies.

The focus of NSAID use has mainly centred on gastrointestinal (GI) side effects and potential cardiovascular toxicity. American, Indian and Japanese manufactures are developing new and innovative NSAID technologies in early attempts at mitigating GI toxicity with oral agents combined with gastroprotective additives. Contemporary technologies coupled with uniquely advanced pharmaceutical manipulations to improve safety and efficacy are important for future therapies. For examples using combination therapies such as: combining the vasodilating agent naproxcinod as the prototypical cyclooxygenase-inhibiting nitric oxide (NO) donor, hydrogen sulfide-releasing compounds to protect GI mucosa; glycoscience technologies combining the intra-articular hyaluronic-acid-

SI-613 (Seikagaku Corporation) with NSAIDs and nano-formulated SoluMatrix™ (iCeutica), submicron technologies that include diclofenac, indomethacin, naproxen, and meloxicam (Atkinson et al., 2013).

To completely avoid or to limit the toxicity of a drug and its side effects are one of the major benefits which can come from nano-therapeutics. To prevent the therapeutic drug from being exposed systemically, and directing the response to a desired organ or cell type, will both improve the overall prognosis and treatment time. A good example of this has recently been seen in a study carried out on the association between cancer and long-term aspirin therapy. A 100mg low dose was administered in healthy (female) volunteers and a statistically noticeable improvement was seen in the common side effects such as gastrointestinal bleeding and peptic ulcers. However no improvements in cancer or colorectal polyps were seen (Cook et al., 2013).

There is more evidence (Coghill et al., 2012) that shows a chemopreventive effect for aspirin and other NSAIDs on colorectal cancer and other cancer types such as prostate cancer (Kawahara et al., 2010), oesophageal (Kang et al., 2013), gastric (Tian et al., 2010) and breast cancer (Brasky et al., 2011, Retsky et al., 2012).

The present study is the only one to our knowledge where the effects of the particle size of these compounds have been investigated at the cellular level in prostate cancer. Our use of lymphocytes is supported by the observations of the WHO/IPCS (Albertini et al., 2000) who reported that lymphocytes are suitable surrogate cells for cancer, and our further work showing that they are suitable surrogates not only for cancer but also for other disease states (Najafzadeh et al., 2012), because DNA is the same in all cells of an individual.

The micronucleus assay (Fenech, 2000) was used for measuring the cytogenetic damage from exposure of our chemicals of interest. The study of DNA damage at the chromosome level is

an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The micronucleus assay has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably and is included in the OECD guidelines. Certain chemicals can be mutagenic or clastogenic and may lead to the induction of micronuclei in cells at the interphase stage. This occurs as a consequence of interference with chromosome structure and/or segregation (Kirsch-Volders et al., 2003, Fenech, 2007, Fenech, 2002).

New research on molecular mechanisms of AD has brought new strategies for therapeutic intervention. Neuroprotection using a combination of NSAIDs, antioxidants and oestrogens could inhibit the early stages of AD. Nanotechnology may prove to be useful in future AD drug delivery strategies, especially drug carrier nano- or microsystems to limit the side-effects of anti-Alzheimer drugs (Di Stefano et al., 2011). Similarly, aspirin may protect from colorectal cancer (Cook et al., 2013) alongside its original role.

Earlier in our studies we demonstrated that DES had a response on blood in the Comet assay. Although DES is a therapeutic compound, it can have unwanted effects due to doses. There is evidence to suggest that an alteration in the prescribed dosing of such therapeutic drugs can have a direct positive effect on patients by decreasing or completely preventing side effects by using a nanotised form to treat prostate cancer. Work carried out by Koong and Watson, (2014) describes the benefits that nano drugs could play on treatments. DES and other pharmaceutical oestrogens have been used at >/= microM concentrations to treat advanced prostate tumours.

5.2 Future work

1. The present work on the genotoxicity of oestrogenic compounds begs the question of whether androgens also have the potential for genotoxicity Therefore, a range of

compounds such as testosterone, dihydrotestosterone and androstenedione should be examined in the Comet and micronucleus assays (in the first instance).

2. In the literature little or no work has been undertaken on nanotising known carcinogens and comparing their effects against bulk versions. Therefore, work should be performed on the nanotisation of carcinogens to determine whether this affects their genotoxicity.
3. Does the administration of nanotised selective oestrogen receptor modulators have an effect on the risk of developing breast or ovarian cancer? Tamoxifen is an extremely useful drug in the treatment of some breast cancer but it has both oestrogenic and anti-oestrogenic properties and is not effective in all circumstances. It would be highly appropriate therefore to test the effect of nanotised tamoxifen on the growth and development of breast and breast cancer cells *in vitro* to assess whether this influenced the effect of tamoxifen on those cells.

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